

Characterization of Root-knot Nematodes (*Meloidogyne* spp.) Associated with *Abelmoschus esculentus*, *Celosia argentea* and *Corchorus olitorius*

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ABSTRACT

Vegetable crops are highly susceptible to a wide range of pests and diseases among which are the root-knot nematodes (*Meloidogyne* species). Traditionally, identification of *Meloidogyne* species had been based on use of morphological characters such as the perennial pattern. In recent times, accuracy of nematode identification using only morphological tools has been challenged due to similarities in morphological characters of some nematode species. The aim of this study was to identify the root-knot nematodes associated with some vegetable crops cultivated on Covenant University farm, Ota, South-west, Nigeria using molecular tools and to determine the population densities of *Meloidogyne* spp. on the selected vegetable crops. Plant-parasitic nematodes were extracted from soil and roots of *Abelmoschus esculentus*, *Celosia argentea* and *Corchorus olitorius* cultivated on Covenant University farm. The nematode species were identified and counted under a compound microscope. The molecular characterization of the *Meloidogyne* species was done using single adult female nematodes and eggs which were picked out for DNA extraction and amplified with specie-specific primers through Polymerase Chain Reaction (PCR) and separated on 0.5% agarose gel. High population of plant-parasitic nematodes was recorded on the vegetable crops cultivated on covenant university farm. Also significantly higher population ($p < 0.005$) of *Meloidogyne* spp. were found in association with *C. argentea* and *Corchorus olitorius* than those recorded on *Abelmoschus esculentus* from the vegetable farm. The molecular characterization of the *Meloidogyne* species from the farm indicates *Meloidogyne incognita* as the nematode species associated with the vegetable crops.

Key words: Root-knot nematodes, vegetable crops, population density, polymerase chain reaction, molecular characterization

INTRODUCTION

In Africa diets, vegetables are important sources of food and medicine (Probst *et al.*, 2010; Padulosi *et al.*, 2011). Farmers have cultivated vegetable crops for generations as additional nutrient sources that supply the body with minerals, vitamins and certain hormone precursors in addition to protein and energy (Mathieu and Meissa, 2007; Wujisguleng and Khasbagen, 2010). Over the years, there has been significant reduction in the production and yield of vegetable crops

due to various pest and diseases among which is the root-knot disease caused by *Meloidogyne* species. Globally, plant-parasitic nematodes have been described as important pests of vegetable and horticultural crops (Johnson *et al.*, 1996; Rius *et al.*, 2007). Damage caused by nematodes accounts for about 40% reductions in quantity and quality of yield of harvested vegetables (Anwar and Mckenry, 2010). These nematodes are predominantly damaging on vegetable crops in tropical and subtropical countries (Sikora and Fernandez, 2005).

Vegetable crops are particularly susceptible to root-knot nematodes which cause the characteristic swelling and disruption of vascular tissues of the plant. They reduce plant vigor, cause root lesions, rotting and deformation. Damage due to the feeding activity of the nematodes on the vegetables could result in the reduction in quality and quantity of the harvested vegetable crops (Schippers, 2000). Affected plants may show slow or stunted growth, yellowing of the leaves, wilting of the plant and seedlings produce few roots. Swelling or galls develop on the root of infected plants as a result of nematode expansion of root cells and damage the vascular tissues of root, thereby interfering with the normal movement of water and nutrients through the plant which eventually lead to reduction in yield or total crop failure.

About ninety species of the genus *Meloidogyne* have been reported worldwide (Hunt *et al.*, 2005), the four most common and frequently occurring species, *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and their known races that readily attack vegetable crops in outdoor as well as in indoor cultivations (Zijlstra *et al.*, 2000) have also been variously reported in association with several cultivated horticultural and vegetable crops in Nigeria (Enopka *et al.*, 1996; CABI and EPPO., 2002; Agwu and Ezigbo, 2005; Bello *et al.*, 2014).

Distinct morphological characters that have been used for identification of *Meloidogyne* spp. includes, the second stage juveniles, morphology of the female adult and males, body length, perineal pattern, head and tail, the excretory pore, dorsal esophageal gland opening, plasmid and spicule (Eisenback and Triantaphyllou, 1991). In recent times, various molecular approaches have been designed for accurate identification of various *Meloidogyne* species. This is primarily because DNA-based methods are rapid and reliable compared to the morphological methods (Powers *et al.*, 2005).

Various methods based on DNA and Polymerase Chain Reaction (PCR) have been developed and successfully used for identifying a large number of *Meloidogyne* species (Singh, 2009). The most popularly used DNA-based methods include: mitochondrial DNA, Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphism DNA (RAPD), Sequence Characterized Region Markers (SCAR-PCR) and Ribosomal DNA (Blok and Powers, 2009).

One of the major challenges facing root-knot nematode taxonomy is the need to establish an accurate, efficient and reliable method of identifying various closely related *Meloidogyne* species. Thus, DNA-based diagnostic methods have gained much acceptance due to their accuracy, reliability and robust nature (Powers, 2004). Besides, they have greater advantage over morphological method of identifying since they can be applied on various stages of development, discriminate individual species from mixed populations and also utilize DNA voucher specimens that have been stored for years (De Ley *et al.*, 2002). This present study seeks to identify the *Meloidogyne* species associated with *Corchorus olitorius*, *Abelmoschus esculentus* and *Celosia argentea* and to determine the population densities of the associated plant-parasitic nematodes of the selected vegetable crops cultivated on Covenant University farm in Nigeria.

MATERIALS AND METHODS

Morphological characterization of *Meloidogyne* spp. of selected vegetable crops

Study site: Soil and root samples were collected from vegetable farm in Covenant University, Ota, Ogun State, Nigeria, located on a latitude of 6°40' 14.37"N and longitude 3°9' 32.31"E.

Sample collection: Soil and root samples were randomly collected from the rhizosphere of the selected vegetables; *Abelmoschus esculentus*, *Celosia argentea* and *Corchorus olitorius* using a hand trowel to a depth of about 15 cm and within a 25 cm radius from the base of the plants. Three replicates per sample were collected in bags and bulked together to form a composite sample which gave a representation of the nematode types and population on the field. Each composite sample was properly labeled and assigned name, collection date, location and time.

Extraction of nematodes from the samples: The Extraction Tray method of Whitehead and Hemming (1965) was employed for the extraction of vermiform nematodes from the samples. Two hundred grams (200 g) each, of the composite samples were placed in the upper sieve of a modified Baermann tray set-up which was made up of a double-ply facial tissue, sandwiched between a pair of plastic sieves and placed in a bowl of water with about 500 mL of water in it. The setup was allowed to remain undisturbed for 24 h, after which the sieves were gently lifted off. The resulting nematode suspension in the bowl were poured into a 500 mL Nalgene wash bottle and left undisturbed for 5 h after which the supernatant was decanted.

Individual adult females and egg mass of RKN were picked from teased galled-root sample under a stereo-microscope and placed PCR tubes containing 8 µL sterile ddH₂O water.

Identification of nematodes: The identification of *Meloidogyne* juveniles in the aqueous suspension was determined using a compound microscope. Two milliliters of the nematode suspension was pipetted after bubbling air through the suspension for homogeneity and dispensed into a counting dish for nematode identification. Nematode identification was done with the aid of pictorial key of Mai and Lyon (1975).

Molecular characterization of *Meloidogyne* spp.

DNA extraction protocols: Two extraction protocols were employed to quantify and ascertain the protocol that yielded higher DNA quality. The protocols are: Qiagen Extraction Kit protocol (Qiagen ®) and a rapid method for nematode identification described by Subbotin *et al.* (2001) was modified for the extraction of DNA from adult females nematodes and eggs.

DNA extraction using qiagen extraction kit: DNA was extracted according to the manufacturer instruction (Qiagen®). DNA Extraction Protocol of Subbotin *et al.* (2001).

Single adult females, juveniles and egg mass were picked alone and were transferred into Eppendorf tubes containing 8 µL distilled water and 10 µL nematode lysis buffer (500 mM KCl, 100 mM Tris-HCl pH 8.0, 15 mM MgCl₂; 1:0 mM DTT, 4.5% Tween 20) and crushed with an microhomogeniser Vibro Mixer (Zürich, Switzerland) for 2.5-3 min. Two microlitres proteinase K were added and the tubes were incubated at 65°C (1 h) and 95°C (10 min) consecutively and finally centrifuged (1 min; 16 000 g). The DNA suspension was stored at -20°C and used for further studies.

Table 1: Root-Knot Nematodes (*Meloidogyne* sp.) primer specific and sequences for PCR reactions

| Primer code | Sequence |
|--|---------------------------------|
| C2F3/1180 | 5'-GGTACAATGTTTCAGAAATTTGTGG-3' |
| Mitochondrial | 5'-TACCTTTGACCAATCAGGCT-3' |
| Far/Rar (<i>M. arenaria</i>) SCAR | 5'-TCGGCGATAGAGGTAATGAC-3' |
| | 5'-TCGGCGATAGACACTACAAACT-3' |
| Fjav/Rjav (<i>M. javanica</i>) SCAR | 5'-GGTGCGCGATTGAACTGAGC-3' |
| | 5'-CAGGCCCTTCAGTGGAACATATAC-3' |
| MI-F/MI-R (<i>M. incognita</i>) SCAR | 5'-GTGAGGATTCAGCTCCCCAG-3' |
| | 5'-ACGAGGAACATACTTCTCCGTCC-3' |

DNA quantification: The concentration and purity of the extracted DNA was monitored using NanoDrop ND-1000 Spectrophotometer at a wavelength of 260 and 280 nm. The DNA was then stored at -40°C until used for DNA-PCR.

Primers for nematode identification: Four *Meloidogyne* species specific primers; C2F3/1108 (mitochondrial), Far/Rar (*M. arenaria*) SCAR, Fjav/Rjav (*M. javanica*) SCAR and MI-F/MI-R (*M. incognita*) SCAR were used in this study. Table 1 shows the primer codes and their sequences.

Polymerase Chain Reactions (PCR): The PCR reactions were carried out in a total volume of 25 µL containing; PCR master mix (12.5 µL), sterilized water (10.5 µL), primer forward (0.5 µL), primer reverse (0.5 µL) and template DNA (1.0 µL). The mixture was placed in a thermal cycler already heated at 94°C. PCR amplification conditions are as follows: denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 70°C for 2 min, repeated for 45 cycles. A 5 min incubation period at 72°C followed the last cycle in order to complete any partially synthesized second strand.

Agarose gel electrophoresis: The PCR reaction products were separated on 0.5% agarose gel with 0.01% ethidium bromide incorporated into the gel. The DNA amplification products were visualized using the automated trans-illuminator (UVP) with digital camera compatible with Window/Vista.

Statistical analysis: The data on nematode population was subjected to analysis of variance and the means partitioned by Duncan's multiple range tests at 5% level of probability.

RESULT

Plant-parasitic nematodes associated with the selected vegetable crops: Root-knot nematodes (*Meloidogyne* species) and some other plant-parasitic nematodes were observed in soil and root samples collected from the selected vegetable crops. Other Plant-Parasitic Nematodes (PPN) found in association with the selected vegetable crops include: *Pratylenchus* sp., *Rotylenchulus* sp., *Scutellonema* sp. and *Helicotylenchus* sp.

Population density of nematodes associated with the selected vegetable crops: The population densities of the *Meloidogyne* species and other parasitic nematodes extracted from the rhizosphere of the selected vegetable crops is presented in Table 2. Significantly higher populations of the *Meloidogyne* species were recorded on the vegetable crops when compared with the

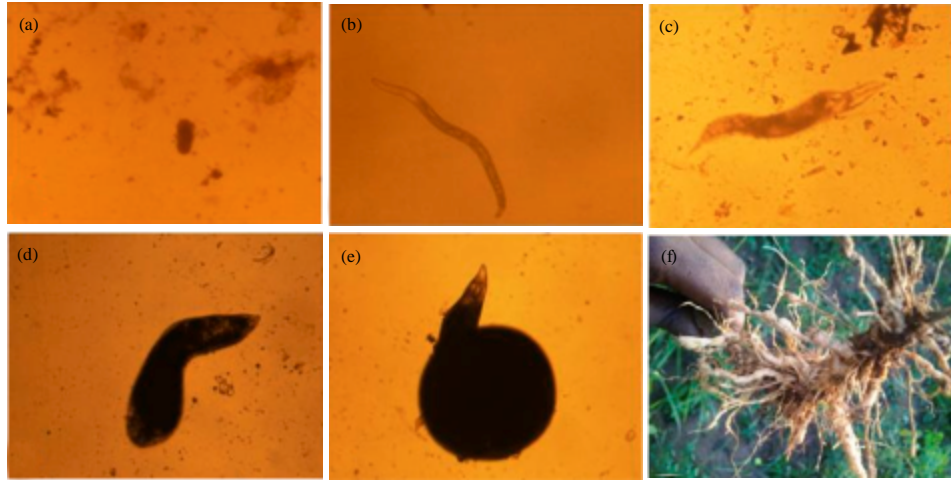


Fig. 1(a-f): Micrographs of the developmental stages and the characteristic root-galling symptom associated with plants infected with of *Meloidogyne* spp., (a) Single egg, (b) 2nd stage juvenile, (c) 3rd stage juvenile, (d) 4th stage juvenile, (e) Adult female and (f) Root galling symptom on *Celosia argentea* root

Table 2: Mean population densities of nematodes associated with vegetable crops on covenant university, ota, farm

| Vegetable crop | <i>Meloidogyne</i> spp./ 200 mL soil | Other Plant-parasitic nematodes/200 mL soil | Non-parasitic nematodes/200 mL soil | Total nematodes/ 200 mL soil |
|-------------------------------|---|--|--|---------------------------------|
| <i>Abelmoschus esculentus</i> | 482.67 | 133.67 | 33.67 | 650.01 |
| <i>Corchorus olitorius</i> | 626.67 | 283.00 | 62.33 | 972.00 |
| <i>Celosia argentea</i> | 642.33 | 249.00 | 72.67 | 964.00 |

Table 3: Mean squares of nematode species associated with vegetable crops in covenant university

| Source of variation | SS | df | MS | F | p-value | F crit |
|---------------------|----------|----|----------|----------|------------|----------|
| Rows | 22481.25 | 2 | 11240.63 | 7.397355 | 0.045295* | 6.944272 |
| Columns | 436921.6 | 2 | 218460.8 | 143.7671 | 0.000188** | 6.944272 |
| Error | 6078.187 | 4 | 1519.547 | | | |
| Total | 465481 | 8 | | | | |

**Significant at $p < 0.001$

population of the other plant parasitic nematodes. The population of root-knot nematodes recorded on *Celosia argentea*, *Corchorus olitorius* and *Abelmoschus esculentus* are 642.33, 626.67 and 482.67, respectively (Table 2 and 3). The result indicates lower populations of RKN on *Abelmoschus esculentus*.

Significantly higher population of plant parasitic nematodes (PPN) were also recorded on the selected vegetable crops than non-parasitic nematodes (Table 2).

Morphological characterization of *Meloidogyne* spp.: Micrographs of the developmental stages *Meloidogyne* spp. obtained from roots of the vegetable crops is described in Fig. 1a-e. The observed developmental stages are single egg, second stage juvenile (J2), third stage juvenile (J3), fourth stage juvenile (J4) and adult female. The characteristic root galling symptom associated with vegetable plants infected with *Meloidogyne* spp. was also shown in Fig. 1f.

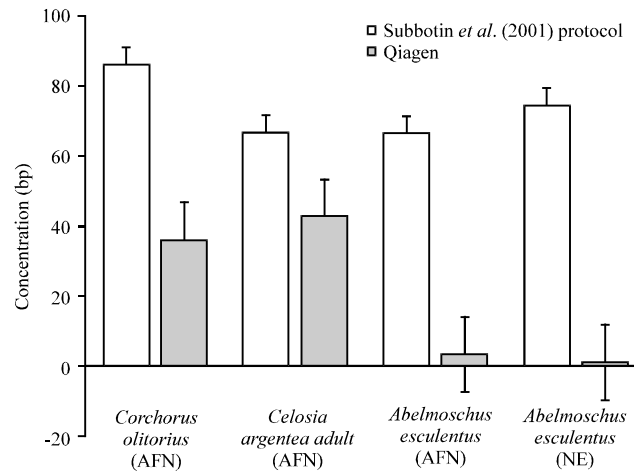


Fig. 2: Comparative DNA concentrations of Subbotin *et al.* (2001) and Qiagen extraction kit protocols

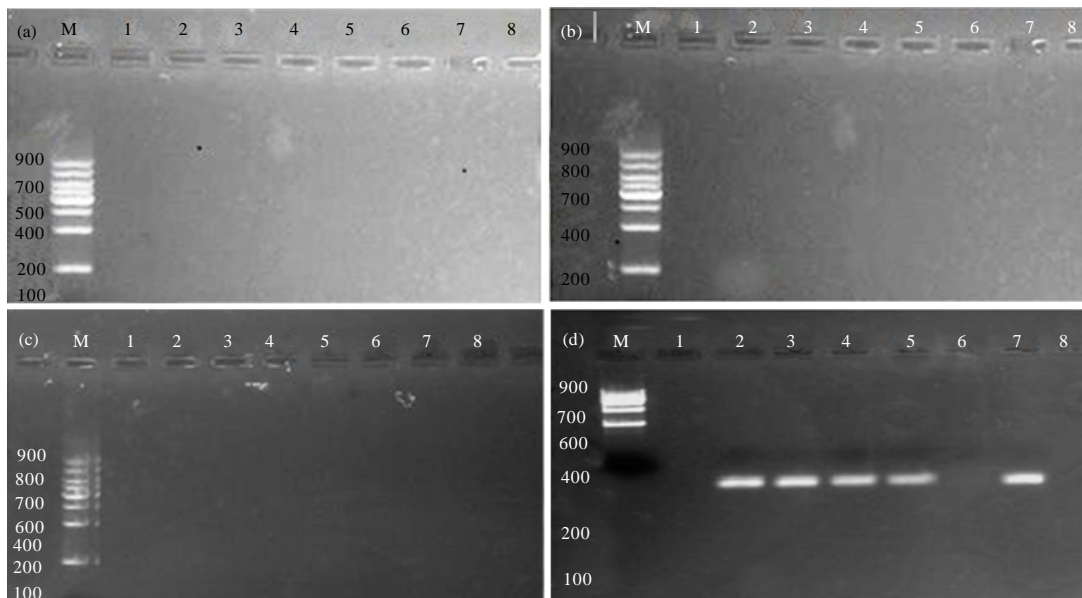


Fig. 3(a-d): Gel electrophoresis of Root- Knot Nematodes (*Meloidogyne* sp.) Species specific primer (a) Far/Rar for *M. arenaria* with no visible band, (b) Fjav/Rjav for *M. javanica* with no visible bands, (c) MIF-MIR for *M. incognita*, bands were visible and (d) Mitochondrial specific primers C2F3/1108 for *Meloidogyne* sp.

DNA quantification and PCR amplification reactions: Modified Subbotin *et al.* (2001) protocol yielded higher concentration of DNA than the Qiagen kit Protocol (Qiagen®). Figure 2 shows the comparative DNA concentrations by both protocols. Primer C2F3/1108 (mitochondrial) generated amplified bands of approximately (400 bp), primers MIF-MIR (*M. incognita*) generated bands of (100-200 bp), no amplified band was observed for both Far/Rar (*M. arenaria*) and Fjav/Rjav (*M. javanica*). Gel electrophoresis of amplified bands is shown in Fig. 3.

DISCUSSION

In this study, Root-Knot Nematodes (*Meloidogyne* species) were found in association with soil and root samples of *Celosia argentea*, *Corchorus olitorius* and *Abelmoschus esculentus* cultivated on Covenant University (CU) Farm, Ota, Nigeria. Similarly, *Pratylenchus* sp., *Rotylenchulus* sp., *Scutellonema* sp and *Helicotylenchus* sp were observed in association with the selected vegetable crops. The higher population of parasitic nematodes recorded on the crops indicate that the selected vegetable crops in Covenant University are highly susceptible and good hosts of root-knot nematodes. Susceptibility of vegetable crops to *Meloidogyne* species has been variously reported worldwide (CABI and EPPO., 2002; Sikora and Fernandez, 2005; Noling, 2012). They have been described as good hosts and highly susceptible to pathogenic nematodes, particularly *Meloidogyne* species (Picard *et al.*, 2004; Presswell *et al.*, 2014; Anwar and Van Gundy, 1989).

In tropical countries, vegetables are usually susceptible to nematode attack and their production depends on the correct management of these pathogens (Sikora and Fernandez, 2005). According to Noling (2012), plant symptoms and yield reductions are often directly related to preplant infestation levels in soil and as infestation levels increases, the amount of damage and yield loss will also increase. The mere presence of these nematodes on the farm is a potential risk to the cultivated vegetables, therefore routine and timely nematode management is essential in order to ensure good plant health and maximize productivity.

The relatively high populations of root-knot nematode recovered in both *Celosia argentea* and *Corchorus olitorius* were indicative of their higher susceptibility to RKN compare to *Abelmoschus esculentus* on CU farm. The presence of various motile stages of developmental stages particularly from infective J2 (second juvenile stage) to adult is an indication that *Meloidogyne* sp. are actively feeding on the roots of the vegetables. These observations are in tandem with the observed stunted growth, chlorotic features at harvest, identification of root galling and brown lesions on the vegetables, particularly, *Celosia argentea* during field observation.

According to Thomas *et al.* (2012) and Kaur and Attri (2013), precision and accuracy in nematode identification is of great importance in order to proffer effective management strategies for nematode control. Identification based on morphological characters is time consuming, labour intensive labor, requires experience and expertise, even then, has not been sufficient for identification and has led to the acceptance of molecular studies.

Rapid and reliable identification of nematode species is required for a number of reasons such as utilizing appropriate crop rotations, managing resistance effectively, developing bio-control strategies, studying virulence, plant-nematode interactions and for quarantine purposes (Zijlstra *et al.*, 2000; Zijlstra *et al.*, 2006).

It was observed in the course of this study that the sensitivity of a DNA extraction assay on nematodes from root and soil extracts requires fresh and live nematode samples. Primers optimized successfully resulted in amplification of DNA obtained from all the nematode sources including egg masses and females. The modified extraction protocol of Subbotin *et al.* (2001) for the extraction of genomic DNA of the *Meloidogyne* species also proved to be more effective than the extraction kit which is more expensive and not readily available to researchers especially in the developing countries.

In this study, the nematode samples tested positive via amplification to the primer C2F3/1108(mitochondrial), there was no amplification for both Far/Rar (*M. arenaria*) and Fjav/Rjav (*M. javanica*) which implies that none of the nematode species belong *M. arenaria* or *M. javanica* but they show amplification for MI-F/MI-R (*M. incognita*) and this suggests that the species of the root-knot nematodes found in association with the selected vegetables crops are *Meloidogyne incognita*.

CONCLUSION

Farmers are often not aware of nematode problems and the damage that nematodes can cause to crops. Covenant university farm was acquired from local settlers in Ota that were predominantly engaged in subsistence agriculture as a means of livelihood. The occurrence of *Meloidogyne* species and other plant-parasitic nematodes in this farmland implies that these nematodes are likely to be wide spread and abundant in the neighboring farmlands. It therefore becomes imperative that resistant plant varieties are cultivated and that effective management strategies are embraced in order to maximize crop yield and also to reduce the spread of nematodes in the local environment.

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